A STUDY OF DELAYED HYPERSENSITIVITY IN LEWIS RATS

By

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INTRODUCTION

Currently there are many unanswered questions in the field of immunology, and particularly in the area of delayed hypersensitivity. In the past decade, much effort has been directed to delineating the similarities and differences between immediate and delayed hypersensitivity. These studies have derived their importance in part from the fact that there is no decisive method of proving the absence of antibody. Hence, the nagging question posed by Karush and Eisen (1) -- whether high affinity antibodies circulating in an undetectable low concentration with continual production might mediate delayed reactions -- remains unanswered.

Many questions are much more susceptible to experimentation. Carrier specificity in delayed hypersensitivity has been demonstrated in guinea pigs. Is this generalization valid in other species? The dispersion of antigen by virtue of molecular weight, route of administration, and dosage has been shown to be a critical determinant of the immune processes which follow. If a greater dosage in sensitization promotes greater dissemination of antigen, and presumably a wider variety of immune processes, what is the net effect on delayed hypersensitivity? The wide use of synthetic polymers of L amino acids, which are not potent antigens themselves, in the preparation of conjugates raises another question. Should one attribute experimental findings to the homogeneity of the carrier, or to its slight potency as an antigen? Do findings in experimental studies of these synthetic polymers have counterparts in studies of native proteins as carriers? Specifically, are highly conjugated proteins non-antigenic as has been shown for the synthetic polymers?

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Currently there are many unanswered questions in the field of immunology, and particularly in the area of delayed hypersensitivity. In the past decade, much effort has been directed to delineating the similarities and differences between immediate and delayed hypersensitivity. These studies have derived their importance in part from the fact that there is no decisive method of proving the absence of antibody. Hence, the nagging question posed by Karush and Eisen (1) -- whether high affinity antibodies circulating in an undetectable low concentration with continual production might mediate delayed reactions -- remains unanswered.

Many questions are much more susceptible to experimentation. Carrier specificity in delayed hypersensitivity has been demonstrated in guinea pigs. Is this generalization valid in other species? The disposition of antigen by virtue of molecular weight, route of administration, and dosage has been shown to be a critical determinant of the immune processes which follow. If a greater dosage in sensitization promotes greater dissemination of antigen, and presumably a wider variety of immune processes, what is the net effect on delayed hypersensitivity? The use of synthetic polymers of L amino acids, which are not immunogenic themselves, in the preparation of conjugates raises another question. Should one attribute experimental findings to the homogeneity of the carrier, or to its slight potency as an antigen? Do findings in experimental studies of these synthetic polymers have counterparts in studies of native proteins as carriers? Specifically, are these conjugated proteins non-antigenic as has been shown for the synthetic polymers?

In the present review of the literature an attempt is made to explore those variables which determine the nature of the immune responses in other species, and to relate their applicability to the present study whose primary topic is delayed hypersensitivity in the Lewis rat. The present study was undertaken to determine whether carrier specificity and the absence of hapten specificity are demonstrable in the delayed hypersensitivity of Lewis rats. The data presented strongly suggest that Lewis rats, unlike guinea pigs, exhibit hapten specific delayed reactions, but no carrier specificity. It remains possible, however, that these differences result from techniques employed in the present study.

REVIEW OF LITERATURE

Of all experimental animals, the guinea pig is the most widely used in studies of delayed hypersensitivity, since there are well established methods for eliciting the delayed response, and since dissociation of immediate and delayed responses can be readily achieved (2,3). Many early studies, moreover, reported that rats and mice failed to develop a delayed tuberculin reaction despite various methods of sensitization (4-9). Indeed, not until 1941 did Wessel (10) demonstrate a delayed cutaneous response in rats one month following intravenous injection of tubercle bacilli. This finding has been confirmed by Rowley *et al.* (11) with pertussis vaccine, and Flax and Waksman (12) using Old Tuberculin.

It is clear that there are definite species differences in the response to antigens. Hemoglobin is a poor antigen in rabbits (13), but is potent both in chickens (14) and guinea pigs (15). Within the Hartley strain of guinea pigs, Levine and Benacerraf found that these animals could be divided into two groups, one of which reacted to conjugates of Poly L lysine as an antigen and the other which could not (16-18). Furthermore, they established that the ability to develop an immune response on exposure to this antigen was inherited as an autosomal Mendelian dominant trait, presumably the ability to degrade the carrier or to undertake a subsequent metabolic operation (19). Maurer noted that polymers of glutamine and lysine were antigenic in rabbits in a ratio of 6:4, in humans in a ratio of 7:3 or 5:5, and in guinea pigs in any of these three ratios (20). He further found that random polymers of

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D alpha amino acids were not antigenic in contrast to L alpha amino acid polymers, and concluded that the ability to metabolize the antigen was a necessary step in the immune response (21).

The age of an animal is a significant variable, and it may be that the capacity to metabolize antigen is related to age. Thus, neonates show an impaired production of antibody (23-27). In a recent review, Silverstein (28) noted that the fetus responds to certain antigens early, and others only later in the course of gestation. Salvin _et al._ (29) observed contact hypersensitivity and allergic encephalomyelitis, both believed to be forms of delayed hypersensitivity, in neonates. Uhr (30) observed delayed hypersensitivity to ovalbumin in neonates, and found that if 1-10 micrograms of antigen in Freud's adjuvant were given during gestation, delayed responses were observed in early neonatal life. These observations suggest that the metabolic pathways necessary for the acquisition and expression of delayed reactivity are present in early life.

Numerous other studies (31-39) have established that large dosages, 15-20 milligrams, of heterologous protein injected intraperitoneally either before or after birth elicit tolerance. Similarly gastric feedings of simple chemicals also establishes tolerance, a specific inability to develop contact hypersensitivity to the hapten ingested (4). Recently Isakovic, Smith, and Waksman (42) suggested that tolerance is induced by the penetration by antigen of the blood-thymus barrier, which is greatest in the adult.

The relationship between the sensitizing dosage and the dissemination of antigen provides a frame of reference for examining immune responses other than tolerance. For example, Salvin and Smith (43,44) showed that

alpha amino acids were not antigenic in contrast to L-alpha amino acid polymers, and concluded that the ability to metabolize the antigen was a necessary step in the immune response (21).

The age of an animal is a significant variable, and it may be that the capacity to metabolize antigen is related to age. Thus, newborns show an impaired production of antibody (25-27). In a recent review, Silverstein (28) noted that the fetus responds to certain antigens early, and others only later in the course of gestation. [illegible] et al. (29) observed contact hypersensitivity and allergic encephalomyelitis, [illegible] believed to be forms of delayed hypersensitivity, in neonates. [illegible] (30) observed delayed hypersensitivity to [illegible] in newborns, and [illegible] in 1-10 [illegible] of [illegible] in Freund's adjuvant were [illegible], delayed responses were observed in early neonatal life. These observations suggest that the metabolic machinery necessary for the acquisition and expression of delayed reactivity are present in early life.

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0.5 microgram of hen egg albumin elicits delayed hypersensitivity in the absence of antibody production.

Molecular weight, moreover, is a significant variable determining the sequestration of antigen in a given tissue. This relationship is well demonstrated by the work of Dresser (41) who found that particles of different molecular weight isolated by centrifugation had quite different antigenic properties. Nossal et al. (46,47) found that while 17 of 144 single cell cultures produced both 7S and 19S antibodies, Salmonella flagellin nonomers escaped the popliteal follicles and elicited predominantly 7S antibodies. In contrast, Salmonella flagella were trapped in the popliteal follicles and produce predominantly 19S antibodies. Thus, the molecular weight is significant in determining the sequestration of antigen in a given tissue. In this regard it is worth noting that a few low molecular weight polypeptides such as glucagon (48), molecular weight 3485, and some synthetic polymers of 3000-5000 molecular weight (20) are antigenic.

Other factors alter the distribution of antigen. The immune state is a case in point; for example, passively transfused antibodies may prevent active sensitization by altering the handling of antigen (49). Adjuvant may provide sustained release and controlled distribution of antigen (50). Since intradermal, subcutaneous, intramuscular, and intraperitoneal injection of antigen in complete adjuvant into guinea pigs elicit delayed hypersensitivity, (51), it would appear that the sustained release is the more critical factor. It is widely held, however, that the intradermal route is preferred in eliciting the delayed response, and that the use of adjuvant enhances both delayed and immediate hypersensitivity (50,53).

Notwithstanding possible differences of species, studies of delayed hypersensitivity in guinea pigs provide perspective for the present study. Various proteins including heterologous proteins and conjugates of homologous and autologous proteins as well as the random synthetic copolymers of L amino acids have been shown to elicit immune responses in the guinea pig.

The contribution of similar amino acid sequence and tertiary structure to the specificity of the delayed response can be seen in the finding that bovine and horse serum albumins cross-sensitize to each other; guinea pigs tested on the ninth day following sensitization with ten microgram doses showed an anaphylactic response to the sensitizing antigen and a delayed response to the related antigen (54). This finding was confirmed by Salvin and Smith (55) who observed cross-reactions among hen, duck, and goose egg albumins.

Even more decisively than these studies of heterologous proteins, the use of conjugates of proteins has demonstrated the marked influence of the carrier protein on the specificity of delayed reactivity. Benacerraf and Gell (2,58) observed that guinea pigs sensitized with 0.1-1.0 micrograms of picrylated proteins in complete adjuvant developed delayed hypersensitivity to the protein carrier, either alone or conjugated to a non-crossreacting hapten. It is of note that in these studies they did not specify the number of haptens per molecule of carrier protein. Gell and Silverstein (57) observed more extensive cross-reactions between ortho, meta, and para isomers of benzoic, benzenesulfonic, and benzenearsonic acid conjugates of the same carrier than those observed in rabbit precipitin or antibody inhibition studies. Thus, the precise nature of the hapten appears to have only slight effect on cross-reactions observed in delayed hypersensitivity.

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It is of importance, however, to note that hapten specificity has been observed in delayed hypersensitivity. Benacerraf and Gell (58) observed that after sensitization with 1 milligram doses, testing with 0.2 milligram of a picryl conjugate of a non-crossreacting carrier elicited a delayed response. Leskowitz (60) has observed that hapten specific delayed hypersensitivity occurs in guinea pigs sensitized with a conjugate of diazotized arsanilic acid to polytyrosine. Positive reactions are observed on testing with conjugates prepared from a wide variety of tyrosine-containing proteins. Further studies (62-63) have confirmed and extended these findings, but have not defined the properties of the hapten responsible for this phenomenon. The importance of these observations of hapten specificity is related to their implication that the "area of recognition" in delayed hypersensitivity need not necessarily involve major portions of the carrier protein.

Homologous and autologous proteins have been used in studying delayed hypersensitivity in two ways, either as denatured proteins or as conjugates. Landsteiner and Chase (64) used conjugates of homologous erythrocyte stromata to elicit contact hypersensitivity. In subsequent work with homologous proteins it has proved difficult to induce contact hypersensitivity (65). Homologous protein conjugates are much less potent antigens than are heterologous proteins coupled to the same hapten (66). Benacerraf and Levine (67) have found that both heavily and lightly coupled conjugates of homologous serum albumen induced delayed hypersensitivity; very significantly, both heavily and lightly coupled antigens cross-reacted yet the sensitizing antigen always evoked the more intense reaction.

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Gell and Benacerraf (54-71) found that heat denatured proteins were as effective as native proteins in provoking delayed hypersensitivity, but do not effect antibody production. In animals sensitized to bovine serum albumin, native or denatured, human serum albumin elicited a delayed response. In a subsequent study (72), animals immunized with alkali denatured autologous gamma globulin developed delayed hypersensitivity to this material, and a few animals immunized with certain forms of denatured autologous gamma globulin reacted to denatured homologous gamma globulin while failing to react with the denatured autologous preparation or the corresponding native homologous preparation. This observation remains unexplained.

One might conclude that the random tertiary structure of denatured proteins diminishes the frequency of collision of antigenic sites with those structural elements and molecules necessary to the acquisition of immediate hypersensitivity and/or antibody-antigen interaction. In contrast whatever molecules and organelles which must combine with antigenic sites to establish and manifest delayed hypersensitivity are clearly able to do so. Studies of the synthetic random polymers of amino acids do not allow evaluation of this hypothesis since their structure is not random; for example, G42L28A30 (glutamine, lysine and alanine in a ratio of 42:28:30) is 40% alpha helix at pH7.5 (73). Indeed, it is clearly established that these polymers, when antigenic, induce both delayed and immediate reactions as do native heterologous proteins; that is, Arthus and delayed reaction are observed concurrently and are not dissociated (22,74-77).

Levine (79) found that lightly coupled benzylpenicylloyl poly L lysine conjugates elicit delayed and immediate reactions, while heavily coupled benzylpenicylloyl conjugates were not antigenic. Similarly, exhaustive succinylation of lightly coupled conjugates rendered them non-antigenic, even though their ability to bind anti-benzylpenicilloyl poly L lysine antibodies was not impaired. A further experiment (18) showed that the lack of antigenicity of exhaustively succinylated conjugates of flourescein poly L lysine could not be attributed to an inability to degrade the polymer, since splenic extracts could do so. Similarly, Kantor and his co-workers (81) reported that the percentage of animals reacting to dinitrophenyl poly-lysine decreased as the degree of conjugation was increased. Other hypotheses which have received support from studies of the antigenicity of amino acid polymers, not duplicated with proteins, include structural rigidity or regularity and the accessibility of antigenic sites as important factors determining the immune responses (82).

The discussion of variables of significance in this study would be incomplete without consideration of the methods of detecting delayed hypersensitivity. The early difficulty in eliciting delayed skin reactivity in the rat has been discussed. It is possible that immaturity may allow the acquisition, but prevent the manifestation of delayed hypersensitivity. Freund (83-84) demonstrated the significance of the age of the animals used; young tuberculous guinea pigs failed to develope delayed skin reactivity to tuberculin, but were as sensitive to systemic shock as were adult tuberculous guinea pigs. Whether or not systemic shock represents delayed hypersensitivity, however, has been questioned (85-86). The test site itself is of some consequence as is seen in the

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failure of intradermal tuberculin to evoke a delayed reaction in the tuberculous chicken unless injected in the comb or wattle (51). Test dosage also is critical, for if too little is used, there will be no demonstrable reaction, and if too much is used, desensitization may result (87). In this study the use of repetitive skin testing raises the question of whether such testing might not also lead to or enhance sensitization. One study utilizing guinea pigs (72) indicated that repeated testing may lead to a low incidence of sensitization. Flax and Waksman, (11), however, noted that repeated skin testing of normal rats with tuberculin (1:10) did not result in sensitization. The question remains whether rats having received complete adjuvant might not become more easily sensitized on skin testing.

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MATERIALS AND METHODS

Animals:

Male Lewis rats weighing 250-300 grams were used throughout for immunization.

Antigens:

Bovine serum albumin, and bovine gamma globulin (Armour Pharmaceutical Company, Kankakee, Illinois). 2,4 dinitrophenyl bovine serum albumin with 40 dinitrophenyl groups coupled to one molecule of albumin (1 to histidine: 39 to lysine), and 2,4 dinitrophenyl bovine gamma globulin with 37 dinitrophenyl groups to one molecule of globulin (4 to histidine: 33 to lysine), kindly prepared by Dr. Z. Ovary.

Immunization:

Each animal was immunized with an emulsion of equal parts of antigen dissolved in saline and complete Freund's adjuvant (8.5 parts Bayol F to 1.5 parts Arlacel A containing 6 mg/ml of illed tubercle bacilli). 0.1 ml of the emulsion was injected into one hind foot pad.

Passive Cutaneous Anaphylaxis:

The method of Binaghi and Benacerraf (88) was employed. 0.1 ml of serum to be tested was injected intradermally into the shaved dorsal skin of normal rats. The animals were injected intravenously 24 hours later with 1 mg of antigen dissolved in 1 ml of 0.5% Evans blue in saline. The animals were killed one hour later and the reactions examined on the inner aspect of the skin.

Passive Hemagglutination:

The protocol used is described by Jankovic, Waksman and Arneson (89). The antisera were inactivated at 56°C for 30 minutes, and absorbed overnight with packed sheep erythrocytes, one drop to one ml. serum. Forminalized sheep erythrocytes were tanned by incubation of equal parts of 2 1/2% erythrocytes in phosphate buffered saline, Ph 7.2, the tanned erythroctes

in a 2 1/2% suspension were treated with 50ml. buffered saline, pH 6.4 containing 7.5mg. antigen. The erythrocytes were washed and suspended in 1% normal serum in buffered saline, pH 7.2. The serum samples were diluted serially and an equal volume of sensitized cells was added to each dilution.

Skin Tests:

Thirty micrograms of the test antigen were injected intradermally in the shaved flank. Arthus reactions were read at 2 to 4 hours, and delayed reactions at 24 and 48 hours. Both average diameter in millimeters and the induration, graded subjectively, were recorded.

Experimental Design:

Ten groups of Lewis rats, each group consisting of three or four animals, were employed in this study. Five groups were sensitized with dosages of 1, 3, 10, 29, and 481 ug of DNPBSA. The remaining five were sensitized with 1, 3, 10, 33, and 530 ug of DNPBGG. Each group was bled and skin tested at 8, 15, and 22 days after sensitization.

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RESULTS

Figures 1 and 2 show that the diameter of the delayed reaction, both at 24 and 48 hours, was directly proportional to the induration. Hence, further reference is made only to the diameter.

As shown in both Figures 3 and 4, the 24 hour reactions observed on the eighth day in animals sensitized with intermediate doses, DNPBSA 29 micrograms and DNPBGG 33 micrograms, exceeded the reactions in animals sensitized with DNPBSA 481 micrograms and DNPBGG 530 micrograms respectively. The delayed responses to sensitization with the intermediate doses attained a peak at eight days, whereas the reactions elicited with the highest sensitizing doses were maximal at 15 to 22 days. By the twenty-second day, the higher sensitizing doses evoked the greater responses. It is of interest that 1 microgram of either antigen failed to sensitize. As seen in Figures 5 and 6, the induration at eight days rapidly diminished by 48 hours, particularly with the highest sensitizing doses. There were no consistent differences between the effects of the two antigens until the twenty-second day when the sensitization with DNPBSA resulted in greater delayed reactions than the corresponding doses of DNPBGG.

In the Arthus reactions depicted in Figures 7 and 8, there was no definite relationship between the dose and response. The greater the sensitizing dosage of DNPBSA, the greater was the Arthus reaction. In contrast, the reaction elicited with the largest dose of DNPBGG was greater than the reaction evoked by the intermediate dose only on the eighth day. Sensitization appeared earlier in the animals sensitized with DNPBGG, but sensitization reached a higher peak by the twenty-second day in animals sensitized with DNPBSA.

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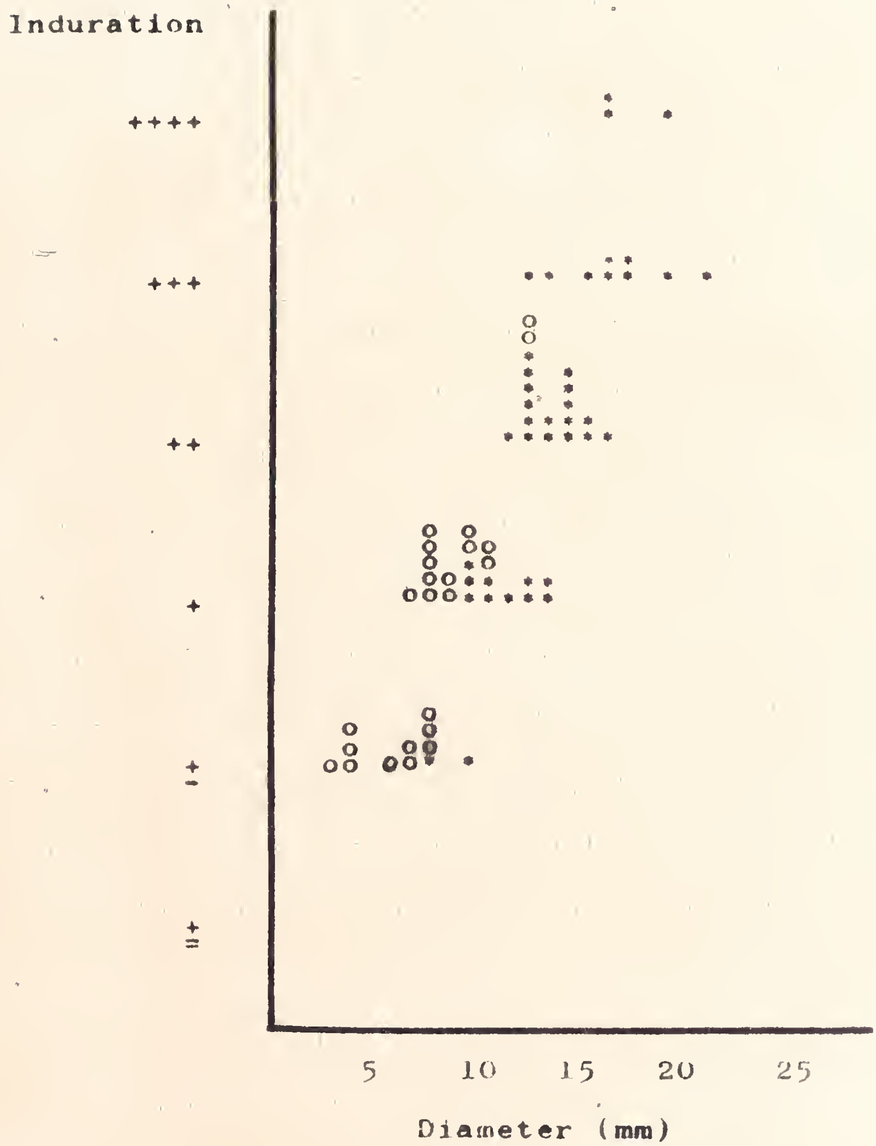
FIGURE 1

DELAYED REACTIONS AT 24 HOURS



* - testing with sensitizing antigen
o - testing with conjugate of non-related carrier

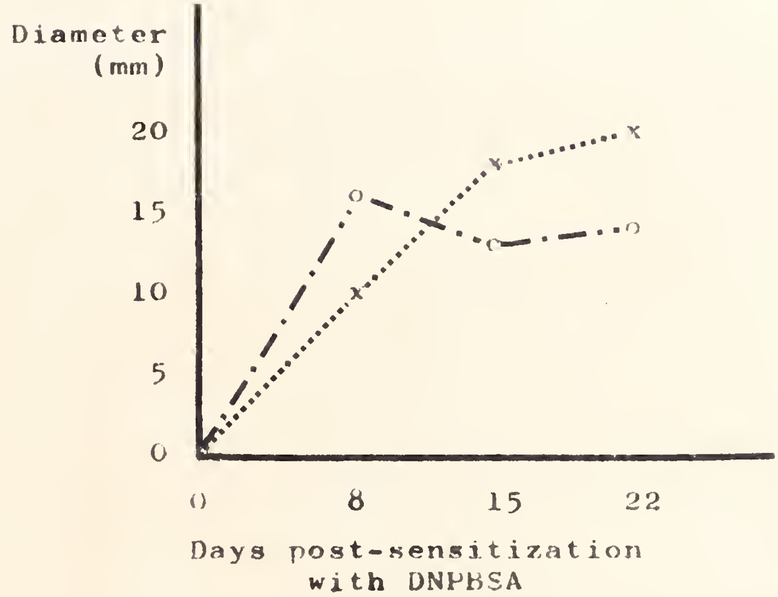
FIGURE 2.

DELAYED REACTIONS AT 48 HOURS

* - reaction to sensitizing antigen

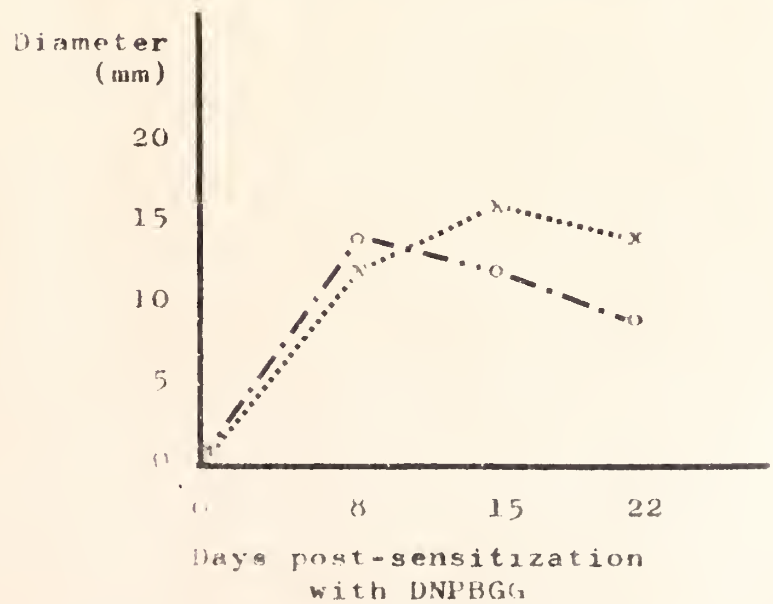
o - reaction to conjugate of non-related carrier.

FIGURE 3. DELAYED REACTIONS AT 24 HOURS



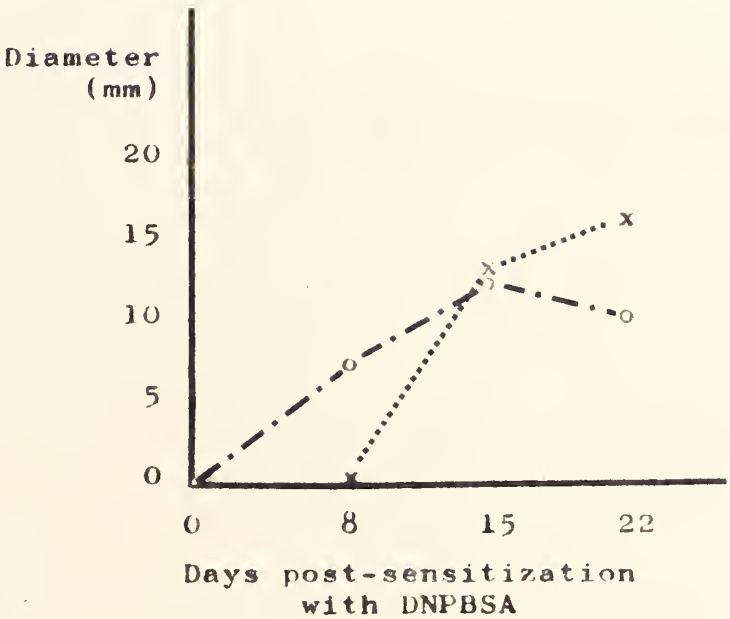
.... - sensitized with 481 ug.
—·—·— - sensitized with 29 ug.

FIGURE 4. DELAYED REACTIONS AT 24 HOURS



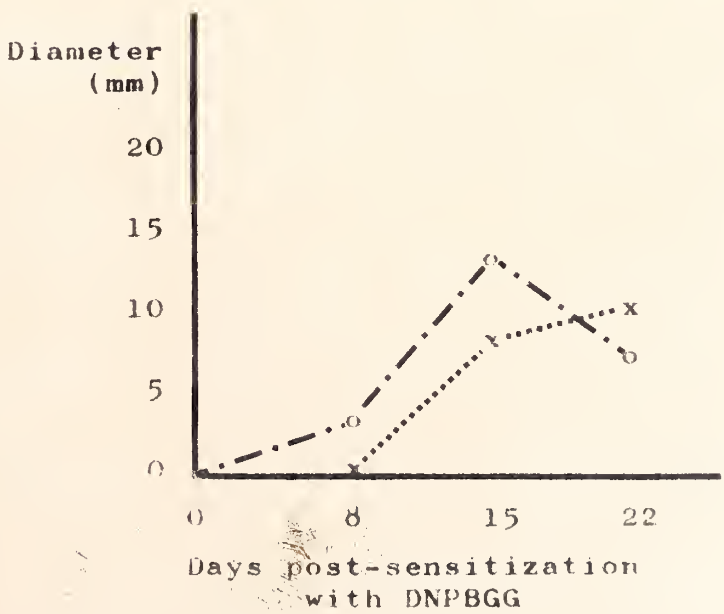
.... - sensitized with 530 ug.
—·—·— - sensitized with 33 ug.

FIGURE 5. DELAYED REACTIONS AT 48 HOURS



.... - sensitized with 481 ug.
—·—·-- sensitized with 29 ug.

FIGURE 6. DELAYED REACTIONS AT 48 HOURS



.... - sensitized with 530 ug.
—·—·-- sensitized with 33 ug.

FIGURE 7. ARTHUS REACTIONS

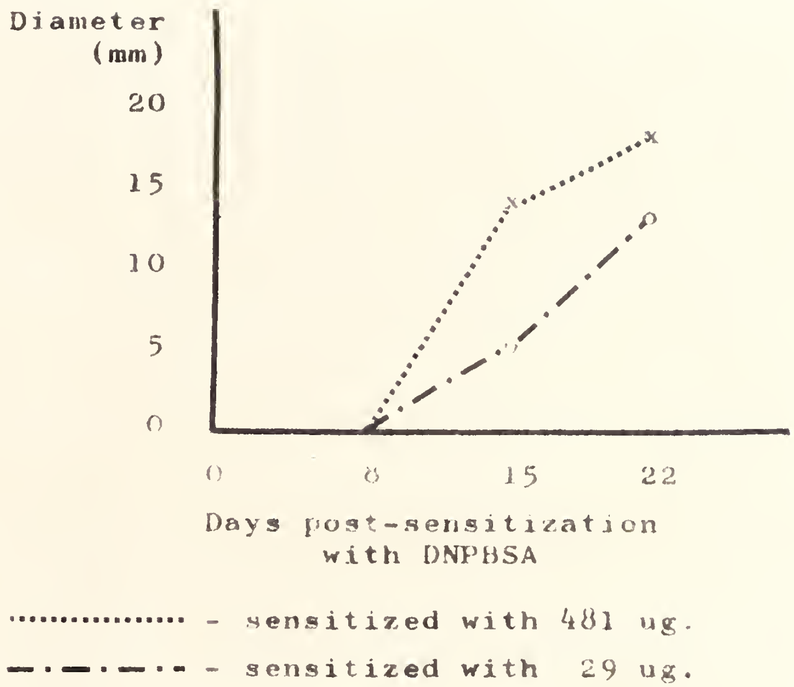


FIGURE 8. ARTHUS REACTIONS

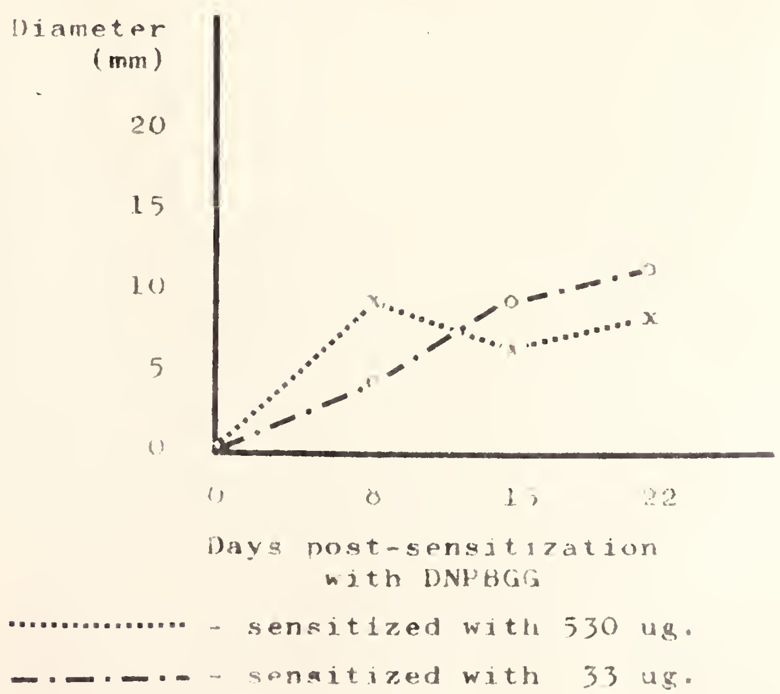


Table 1. Cutaneous Reactions. (Average Diameter in mm.).

Sens. Ag.	Day	# of rats	DNPBSA 30 ug			DNPBGG 30 ug			BSA 30 ug.		
			(Arth.	24 hr.	48hr.)	(Arth.	24hr.	48 hr.)	(Arth.	24hr.	48hr.)
DNPBSA 481 ug	8	4	0	10	0	0	0	0	0	0	0
	15	3	14	18	13	1	10	5	0	0	0
	22	3	18	20	16	11	9	9	0	0	0
29 ug	8	3	0	16	7	0	0	0	0	0	0
	15	3	5	13	12	0	5	5	0	0	0
	22	3	13	14	10	7	3	3	0	0	0
1 ug	8	3	0	0	0	0	0	0	0	0	0
	15	3	0	0	0	0	0	0	0	0	0
	22	3	0	0	0	0	0	0	0	0	0

Table 2. Cutaneous Reactions. (Average Diameter in mm.)

Sens. Ag. DNPBGG	Day	# of rats	DNPBGG 30 ug. (Arth.	24hr,	48hr.)	DNPBSA 30 ug. (Arth.	24hr.	48hr.)	BGG 30 ug. (Arth,	24hr.	48hr.)
530 ug	8	4	9	12	0	0	0	0	0	0	0
	15	4	6	16	8	13	8	4	0	0	0
	22	4	8	14	10	8	10	6	0	0	0
33 ug	8	4	4	14	3	0	0	0	0	0	0
	15	4	9	12	13	9	9	9	0	0	0
	22	4	11	9	7	6	4	3	0	0	0
1 ug	8	4	0	0	0	0	0	0	0	0	0
	15	3	0	0	0	0	0	0	0	0	0
	22	3	0	0	0	0	0	0	0	0	0

Table 3. Cross-reactions Observed in Passive Cutaneous Anaphylaxis.

Sens. Ag. DNPBSA	Day	No. Positive DNPBSA	Diameter* (avg.mm.)	No. Positive DNPBGG	Diameter* (avg.mm.)	Avg. Passive Hemaggl. Tube
481 ug	8	1/4	23	0/2	0	2
	15	3/4	10	0/1	0	4
	22	3/3	18	0/3	0	4
29 ug.	8	1/3	31	1/2	16	0
	15	2/3	23	1/2	16	5
	22	2/3	38	2/3	15	6
1 ug.	8	0/4	0	0/4	0	1
	15	0/4	0	0/4	0	0
	22	0/4	0	0/4	0	0

* Average Diameter of Positive Reactions.

Table 4. Cross-reactions Observed in Passive Cutaneous Anaphylaxis

Sens. Ag. DNPBGG	Day	No. Positive DNPBGG	Diameter* (avg. mm.)	No. Positive DNPBSA	Diameter* (avg.mm.)
530 ug.	8	2/4	14	2/4	12
	15	2/4	14	0/4	00
	22	3/4	24	3/4	21
33 ug.	8	0/4	0	1/4	12
	15	3/4	21	3/4	17
	22	4/4	25	4/4	21
1 ug.	8	0/4	0	0/4	0
	15	0/4	0	0/4	0
	22	0/4	0	0/4	0

* Average Diameter of Positive Reactions.

Table 5. Cutaneous Reactions 15 Days Post-Sensation

Sens. Ag.	DNPBSA 30 ug.			DNPBGG 30 ug.		
	Arth.	24 hr.	48 hr.	Arth.	24 hr.	48 hr.
DNPBSA 10 ug.	6±	7±	6±	0	0	0
	7±	8±	10±	0	0	0
	0	0	0	0	0	0
DNPBSA 3 ug.	6±	0	0	0	0	0
	0	0	0	0	0	0
	5±	0	0	0	0	0
DNPBGG 10 ug.	12+	10+	7±	14+	17+++	16+
	12+	10±	10±	12+	19+++	15++
	12+	7±	6±	11+	14++	13+
DNPBGG 3 ug.	10+	7±	0	10+	15+	12±
	0	0	0	0	13+	12+
	13+	8±	7±	13+	12+	13+

Note: All animals had been skin tested 8 days post-sensitization.

In contrast to previous work demonstrating carrier specificity and the absence of hapten specificity in guinea pigs, the data shown in Table 1 and 2 indicate hapten specificity in the absence of carrier specificity. The carrier alone failed to elicit a delayed response in animals sensitized with either conjugate. In both cases, moreover, delayed reactions, the intensity of which varied directly with the sensitizing dosage, were observed upon skin testing with dinitrophenyl conjugated to the non-related carrier at 15 days, but not at 8 days. The hapten specific response was less than the reaction elicited by testing with the sensitizing antigen.

Tables 3 and 4 illustrate that antibodies producing passive cutaneous anaphylaxis were present as early as the eighth day, and reacted with the dinitrophenylated, non-related carrier. Table 3 also reveals that there was no correlation of passive cutaneous anaphylaxis with either passive hemmagglutination or Arthus reactions.

Table 5 presents in detail data establishing that the sensitizing antigen contained the conjugated non-related carrier in insufficient amounts to cause sensitization, and thus give rise to apparent hapten specific delayed reactions. It is apparent that the delayed reaction observed in animals sensitized with 3 micrograms of DNPBGG, and tested with DNPBSA, was approximately equal to the reactions seen in animals sensitized with DNPBSA 10 micrograms, and tested with DNPBSA. Since 10 micrograms of DNPBSA could not be contained in 3 micrograms of DNPBGG, contamination with the conjugated, non-related carrier could not have produced these results.

In contrast to previous work demonstrating hapten specificity and the absence of hapten specificity in guinea pigs, the data shown in Table 1 and 2 indicate hapten specificity in the absence of carrier specificity. The carrier alone failed to elicit a delayed response in animals sensitized with either conjugate. In most cases, moreover, delayed reactions, the intensity of which varied directly with the sensitizing dosage, were observed upon skin testing with dinitrophenyl conjugated to the non-related carrier at [illegible] days, but not at 5 days. The hapten specific response was less than the reaction elicited by testing with the sensitizing antigen.

Tables 3 and 4 illustrate that antibodies producing passive cutaneous anaphylaxis were present as early as the [illegible] day, and reacted with the dinitrophenylated, non-related carrier. Table 4 also reveals that there was no correlation of passive cutaneous anaphylaxis with either passive hemagglutination or Arthus reaction.

Table 5 presents in detail data establishing that the sensitizing antigen contained the conjugated non-related carrier in insufficient amount to cause sensitization, and thus give rise to apparent hapten specific delayed reactions. It is apparent that the delayed reaction observed in animals sensitized with 1 microgram of [illegible], and tested with [illegible], are approximately equal to the reactions seen in animals sensitized with [illegible] 10 micrograms, and tested with [illegible]. Since the quantity of [illegible] would be contained in 1 microgram of [illegible], contamination with the conjugated non-related carrier could not have produced these results.

DISCUSSION

The effects of the sensitizing dosage, molecular weight, and nature of the antigen are well illustrated in these data. Uhr et al. (3) demonstrated that, using antigen-antibody complexes, the amount of antigen required for maximal delayed sensitization of guinea pigs was between 0.1 and 1 micrograms, whereas much larger amounts were required for maximal antibody production. Hence, it is not surprising that intermediate dosages produced at eight days responses as large as did the highest dosages; the fact, however, that the intermediate doses evoked greater responses than the highest doses requires comment. It is apparent that the excess of antigen produces either the suppression of the activity of those cells or their products responsible for the delayed hypersensitive state, or activation of antagonistic responses in other organs and tissues. The former hypothesis is somewhat strengthened by the fact that induration at eight days is short-lived, particularly with the highest dosage and in the animals sensitized with DNPBSA. It is probable that the number of particles or molecules, which is obviously greater with the larger doses and greater per microgram of DNPBSA than DNPBGG, is the critical variable.

If the release of antigen from the adjuvant emulsion is sustained at higher levels over the interval of this experiment in those animals sensitized with the highest dosages, and if the continuous presence of antigen is necessary for the maintenance of the delayed hypersensitive state, it necessarily follows that the delayed reactions at 22 days will be greater in the animals receiving the higher sensitizing dosage. The data fulfill this prediction. At 22 days the animals sensitized with DNPBSA give larger reactions than those sensitized with DNPBGG.

DISCUSSION

The effects of the sensitizing dosage, molecular weight, and nature of the antigen are well illustrated in these data. Uhr et al. (3) demonstrated that, using antigen-antibody complexes, the amount of antigen required for maximal delayed sensitization of [illegible] was between 0.1 and 1 microgram, whereas much larger amounts were required for maximal antibody production. Hence, it is not surprising that intermediate dosages produced at eight days [illegible] did the highest dosages; the fact, however, that the intermediate doses evoked greater responses than the highest [illegible]. It is apparent that the excess of antigen [illegible] of the activity of those cells or their products responsible for the delayed hypersensitive state, or [illegible] in other organs and tissues. The former hypothesis [illegible] by the fact that induration at eight days [illegible] with the highest dosage [illegible]. It is probable that the number of particles or molecules, [illegible] greater with the larger doses [illegible] ONPBGG, is the critical variable.

If the release of antigen from the [illegible] higher levels over the interval of this experiment in those animals sensitized with the highest dosages, and if the continuous presence of antigen is necessary for the maintenance of the delayed hypersensitive state, it necessarily follows that the [illegible] be greater in the animals receiving the [illegible]. [illegible] data fulfill this prediction. [illegible] ONPBSA give larger reactions than those sensitized with [illegible].

Albumins are less potent antigens in guinea pigs (85), and probably also in Lewis rats (89.90). At eight days Arthus reactions were greater in DNPBGG sensitized animals than in DNPBSA sensitized animals. The greater duration of immune responses elicited with DNPBSA is perhaps explained by its sustained release from the adjuvant. This statement is contingent upon the fact that there are more molecules per microgram of DNPBSA than in DNPBGG, and upon the assumption that the rate of release of antigen from adjuvant is proportional to the concentration of molecules of antigen while holding the adjuvant surface area constant.

The delayed reactions observed at eight and fifteen days support the hypothesis that a larger site of recognition is involved in delayed reactions than immediate reactions; the data, nonetheless, represent a departure from the previous findings in guinea pigs that the carrier protein itself will provoke a delayed reaction in animals sensitized with conjugates. The cutaneous reactions observed at eight days suggest that the site of recognition involved in the delayed reaction involves both the hapten and the carrier; neither alone elicits a reaction.

One cannot attribute the disparity between these data and published data to difference in sensitizing dose or challenge dose. Benacerraf and Gell (2,58) used picrylated bovine gamma globulin in amounts of 0.1 to 100 micrograms to sensitize guinea pigs, and found that skin testing with bovine gamma globulin in doses of 3-10 micrograms evoked a delayed reaction.

A second possible explanation is that the heavy conjugation of the carrier protein prevented the degradation or metabolism of the sensitizing antigen in sites responsible for the production of delayed hypersensitivity,

[illegible] and less potent antigens in guinea pigs (90), and probably also in Lewis rats (89,90). At eight days strong reactions were greater in DNP-BGG sensitized animals than in DNP-HSA sensitized animals. The greater duration of immune responses elicited with DNP-HSA is perhaps explained by its sustained release from the adjuvant. This statement is contingent upon the fact that there are more molecules per surface of DNP-HSA than in DNP-BGG, and upon the assumption that the rate of release of antigen from adjuvant is proportional to the concentration of molecules of antigen while holding the adjuvant surface area constant.

The delayed reactions observed at eight and fifteen days support the hypothesis that a larger site of recognition is involved in delayed reactions than immediate reactions; the data, nonetheless, represent a departure from the previous findings in guinea pigs that the carrier protein itself will provoke a delayed reaction in animals sensitized with conjugates. The cutaneous reactions observed at eight days suggest that the site of recognition involved in the delayed reaction involves both the hapten and the carrier; neither alone elicits a reaction.

One cannot attribute the disparity between these and previous published data to difference in sensitizing dose or challenge dose. [illegible] and Gell ([illegible],35) used nitrophenol bovine gamma globulin in [illegible] to 100 micrograms to sensitize guinea pigs, and found [illegible] with bovine gamma globulin in doses of 1-10 [illegible] elicited a delayed reaction.

A second possible explanation is that the rapid catabolism of the carrier protein prevented the degradation or metabolism of the [illegible] which is responsible for the recognition of delayed hypersensitivity.

without adversely affecting its metabolism in sites producing antibody. No one, however, has reported that increasing the number of haptens extinguishes the delayed reactivity first, and that with further increments in the number of haptens per molecule of antigen, both immediate and delayed responses are lost.

A third hypothesis is that the random process of conjugation leaves small amounts of unconjugated and very lightly conjugated carrier protein which are sufficient to sensitize the guinea pigs. It should be noted that Salvin (22) demonstrated that very small amounts of antigen could sensitize guinea pigs in the absence of antibody. Furthermore, the cutaneous reaction of delayed hypersensitivity is less easily induced in the rat.

The presence of hapten specificity in the delayed reactions upon testing at fifteen and not at eight days raises the question whether this finding is a consequence of earlier skin testing. Skin testing at eight days with the dinitrophenylated, non-related carrier would seem unlikely to result in sensitization, since the intensity of the hapten specific delayed reactions was directly proportional to the sensitizing dosage. Furthermore, as was noted earlier, repeated skin testing with old tuberculin did not sensitize rats. Therefore, one may conclude that skin testing with the conjugate of the non-related carrier did not result in sensitization.

Since the acquisition of hapten specificity in the delayed reactions is not observed until serum antibody is present, it is possible, but unlikely, that the hapten specific reactions are mediated by antibody, and do not represent delayed hypersensitivity. Passive transfusion of serum would be necessary to establish that these reactions are not mediated by antibody. Nonetheless, it remains highly probable that the delayed appearance of

hapten specificity at 15 days represents true hapten-specific delayed reactions.

SUMMARY

Six groups of Lewis rats were sensitized with varying doses of dinitrophenyl conjugated to either bovine serum albumin or bovine gamma globulin. Each group was skin tested eight, fifteen, and twenty-two days after sensitization.

Sensitizing doses of DNPBSA 29 micrograms and DNPBGG 33 micrograms provoked greater delayed reactions at eight days than doses of DNPBSA 481 micrograms and DNPBGG 530 micrograms respectively. The higher sensitizing doses produced greater reactions by the twenty second day.

Delayed reactivity to the carrier of the sensitizing conjugate was consistently absent. Delayed reactions were elicited by testing with dinitrophenyl conjugated to the non-related carrier.

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